GLUCOSE, GLUCONATE, AND 2-KETOGLUCONATE OXIDATION BY ACETOBACTER MELANOGENUM*

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During recent years there has been renewed interest in the course of oxidation of glucose via gluconic acid, both because it provides a mechanism for pentose formation (1) and because it appears to represent an alternate source of trioses (2, 3) considered heretofore to originate only by way of the reactions commonly referred to as the Embden-Meyerhof mechanism. Present evidence suggests that glucose may be oxidized to gluconic acid, and this in turn to 2- or 3-ketogluconic acid. Decarboxylation of the latter substances then yields pentose; trioses may arise from pentose by a C_2—C_3 split (3), or directly from gluconic acid by a C_3—C_3 split, as reported by Entner and Doudoroff (2).

Direct oxidation of sugars has been found with molds, *Pseudomonas* sp., and, particularly, *Acetobacter* sp. Studies with *Acetobacter* sp. have shown the existence of oxidative patterns which often lead to the accumulation of large amounts of incomplete oxidation products such as gluconic and 5-ketogluconic acids, which can be readily manufactured on a large scale with the aid of *Acetobacter gluconicum* and *Acetobacter suboxydans*.

Another member of this group, *Acetobacter melanogenum*, has not yet been investigated in this respect in sufficient detail. That it carries out incomplete oxidation is indicated by its failure to oxidize ethanol beyond the stage of acetic acid and by the fact that it produces dihydroxyacetone from glycerol. Particular interest attaches to this species, however, because its sugar metabolism appears to be peculiar. It is known that cultures in glucose solutions, both in yeast extract (4) and in synthetic medium (5), always turn a dark brown in time; this has never been observed with cultures in which glucose is replaced by ethanol or glycerol. The brown discoloration therefore seems to be directly associated with glucose oxida-

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tion, indicating an unusual type of degradation. For this reason detailed studies were initiated.

Methods and Materials

A. melanogenum strain MA 6.2 of the Hopkins Marine Station culture collection was used. It was routinely grown in a medium composed of 10 per cent yeast autolysate, 2 per cent glucose, and 0.36 per cent $CaCO_3$ in tap water. The medium, sterilized in 500 ml. amounts in Fernbach flasks, was inoculated with a suspension of cells from yeast-glucose-$CaCO_3$ agar slants and incubated at 28° for 48 hours on a rotary shaker. After this period little, if any, $CaCO_3$ remained in the solution, and the cells were harvested by centrifugation, washed once in tap water, and used as suspensions in 0.05 M or 0.01 M phosphate buffer at pH 6.0, or in distilled water, adjusted to pH 3.5. The density of the suspensions used for manometric experiments corresponded to a Klett reading of approximately 400 with a 660 mμ filter. 2 ml. aliquots of such suspensions were used per vessel.

Cell-free extracts were prepared by the method of McIlwain (6). After washing and concentrating by centrifugation, the cells were ground with slightly more than twice their wet weight of levigated alumina powder and mixed with about 6 parts of 0.05 M phosphate buffer at pH 6.0. The suspension was spun in a Servall centrifuge in the cold at about 12,000 r.p.m. for 5 to 10 minutes. The supernatant liquid was used at once, or frozen and stored for later use. All manometric experiments were performed with the standard Warburg apparatus.

Results

Oxidations by Resting Cell Suspensions—Washed cell suspensions of *A. melanogenum* invariably had a negligible endogenous metabolism. The addition of glucose resulted in a rapid O_2 consumption, the amount used per mole of glucose depending upon the age of the cells (Fig. 1). With freshly harvested cells from a young culture, O_2 uptake approached 2 moles per mole of glucose, and was accompanied by the liberation of about 1 mole of CO_2. Cells that had been stored for 24 to 72 hours at low temperatures generally took up only about 1.5 moles of O_2 per mole of glucose, and produced little CO_2 in the process. Similar results were obtained with fresh cell suspensions in the presence of 1.25×10^{-3} M 2,4-dinitrophenol (DNP).

The results suggest that the metabolic activities of older cells are more restricted than those of young cells, as has also been found for *A. suboxydans* (7, 8). The data on O_2 utilization and CO_2 production for old cell suspensions are compatible with an oxidation of glucose to saccharic acid or to a diketo- (or aldehydoketo-)gluconic acid. Since it was found that a

solution in which all of the glucose had been oxidized contained substances that reduce alkaline copper reagent in the cold, and that, per mole of glucose oxidized, only one carboxyl group is generated (see below), saccharic acid can be ruled out as the major oxidation product, leaving a conversion according to the equation:

(1) $$\text{Glucose} + 1.5O_2 \rightarrow \text{diketogluconic acid}$$

as the more probable.

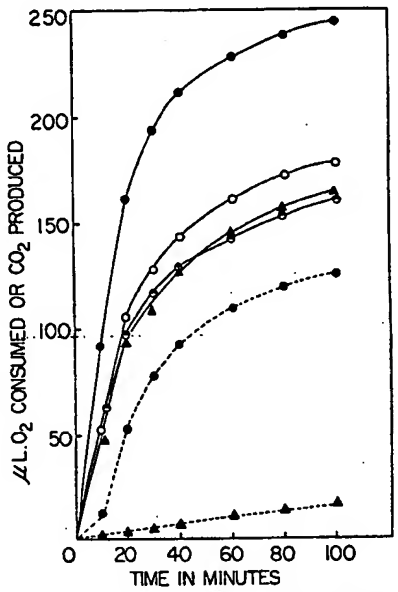


FIG. 1. Oxidation of glucose by *A. melanogenum*, as influenced by the age of the cells and DNP. The vessels contained 2 ml. of cell suspension in 0.05 M phosphate, pH 5.9; 5 μM of glucose; 0.3 ml. of 10^{-2} M DNP or water; total volume, 2.4 ± 0.2 ml. 10 per cent KOH in the center well; endogenous values not subtracted. Solid line, oxygen; dash line, carbon dioxide; ●, freshly harvested cells; ○, cells after 3 days at 4°; ⊖, fresh cells + DNP; ▲, aged cells + DNP.

On the basis of present biochemical concepts, this mode of oxidation would require that gluconic and ketogluconic acids be intermediate products. Gluconic acid is readily oxidized, as is 2-ketogluconic acid,[1] especially at pH 3.5 where the oxidation proceeds faster than at pH 6, probably owing to a better penetration of the undissociated acids into the cells. Oxidation of 5-ketogluconic acid could not be detected.

Suspensions of "old" cells consumed approximately 1.0 and 0.5 moles of

[1] We should like to thank Dr. F. H. Stodola and Dr. P. Regna for the Ca 2-ketogluconate used in this investigation.

O_2 per mole of gluconic and 2-ketogluconic acids, respectively. With suspensions of freshly harvested "young" cells, the amounts of O_2 utilized were larger and CO_2 was evolved.

The production of an acid during the oxidation of glucose was demonstrated with suspensions in dilute bicarbonate solutions by measuring residual bicarbonate at regular intervals, as described by Stokes (9). For this purpose suspensions in 0.06 per cent $NaHCO_3$ were used; the gas phase consisted of a mixture of 20 per cent CO_2 in air. These experiments showed that 1 acid equivalent was formed from glucose; no acid production could be detected when gluconic or 2-ketogluconic acid was used as substrate.

TABLE I

*Acid Production from Glucose As Related to Oxygen Consumption by Intact Cells of A. melanogenum**

	Acid as CO_2 liberated from $NaHCO_3$	Oxygen uptake	O_2:CO_2
min.	*μl.*	*μl.*	
0	0	0	0
10	69	83	1.20
20	97	119	1.23
30	104	129	1.24
40	112	142	1.27
50	110	142	1.29

* The vessels contained 2 ml. of cell suspension in 0.06 per cent $NaHCO_3$ equilibrated with 20 per cent CO_2-air mixture, 0.1 ml. (5 μM) of glucose, 0.4 ml. of 10^{-2} M DNP, gas phase, 20 per cent CO_2 in air, and 0.2 ml. of 10 per cent H_2SO_4 tipped in at 10 minute intervals.

Studies on the oxidation of glucose to 5-ketogluconic acid by *A. suboxydans* (8) have indicated that here the oxidation proceeds in a clearly defined stepwise manner, all of the glucose being first converted to gluconic acid with a subsequent oxidation of the latter to 5-ketogluconic acid. If the oxidation by *A. melanogenum* were to follow this pattern, the ratio of O_2 consumed to acid produced should be 0.5 during the early phase of the process. As shown by the data of Table I, this is not the case; even as early as 10 minutes after the addition of glucose, when only 63 per cent of the maximal amount of acid had been formed, 2.4 times the corresponding theoretical amount of O_2 had been utilized. This implies that gluconic acid was further oxidized in the presence of residual glucose, a phenomenon also observed by Stubbs *et al.* (10) in their studies on the oxidation of glucose to 2-ketogluconic acid by *Pseudomonas* sp.

Further evidence in support of this contention was provided by paper chromatographic analysis of glucose solutions partially oxidized by suspen-

sions of *A. melanogenum*. For this purpose, cell suspensions in 0.01 M phosphate buffer, or in distilled water, to reduce salt effects during chromatography to a minimum, were shaken in the presence of 10 μM of glucose per ml., and samples were removed at regular intervals over a period of 2 hours. The cells were spun down and amounts of supernatant liquid corresponding to 40 to 50 γ of substrate (0.02 to 0 03 ml.) were placed on Whatman No. 1 filter paper and dried. Separation of components was achieved with *n*-butanol-acetic acid-water ((11) Solvent A), and the chromatogram developed with brom phenol blue or *p*-anisidine hydrochloride (12) in butanol. Glucose, ribose, lactose, arabinose, and xylose, gluconic, 2- and 5-ketogluconic, saccharic, glutaric, α-ketoglutaric, glucuronic, ascorbic, pyruvic, and kojic acids, glyoxal, dihydroxyacetone, glucuronolactone, and gluconolactone were used as reference compounds.

It was found that even after 5 minutes incubation the solutions contained, besides much residual glucose, a substance yielding a pink-purple spot with *p*-anisidine, identical in color and position to that produced by 2-ketogluconic acid. Soon thereafter a second product appeared; its behavior did not correspond to that of any of the reference compounds. It moved with an R_F value slightly smaller than that characteristic for 2-ketogluconate, produced a yellow spot with *p*-anisidine, and was acidic towards brom phenol blue. In the course of time the intensity of the glucose spot diminished and that of the ketogluconate spot remained fairly constant, while that of the yellow spot increased. After 2 hours only the last one remained.

Similar experiments with gluconic acid instead of glucose as a substrate showed that here, too, 2-ketogluconic acid and the final product, producing the yellow spot, were present at the same time, and appeared practically from the start. Tests with 2-ketogluconic acid revealed its gradual disappearance, and replacement by the same end-product. Other solvent systems used in following the stepwise course of the reaction by means of paper partition chromatography were ascending water-saturated phenol (Solvent B) and descending water-saturated isobutyric acid (Solvent C). With aniline hydrogen oxalate (13) as a developing agent, the unknown product appeared as a green-yellow spot which did not fluoresce under the ultraviolet lamp. With descending Solvent C, the system adopted for routine analysis of reaction mixtures, after 24 hours flow, typical R_G values (the ratio of the distance the substrate traveled with respect to glucose) were 2-ketogluconate 0.85, unknown compound 0.59.

From the above observations it was concluded that the oxidation of glucose does not lead to a preliminary accumulation of the corresponding amount of gluconic or 2-ketogluconic acid, but that the oxidation of these two intermediate products occurs concomitantly with the production of the

unknown acid. The fairly constant and high ratio of O_2 used to acid produced (Table I) further implies that not even ketogluconic acid accumulates to an appreciable extent. It seems probable that these results must be ascribed to the very low glucose concentrations used (2 per cent or less); in the presence of high substrate concentrations the picture is quite different, and large amounts of gluconic acid can be isolated from a culture of *A. melanogenum* in yeast extract with 10 per cent glucose and 3 per cent $CaCO_3$.

Some observations on O_2 uptake and CO_2 production by suspensions of "young" cells (*e.g.*, Fig. 1) suggested that such organisms may carry out a further single step oxidation of the diketogluconic acid to a ketopentonic acid. Special experiments on the quantitative relations between substrate oxidized, O_2 consumed, and CO_2 produced suggest that the "young" cells carry out a much more complicated oxidation, which is accompanied by a considerable oxidative assimilation of substrate-carbon. This particular phase has not been pursued further.

Oxidations by Cell-Free Extracts—Extracts of alumina-ground cells of *A. melanogenum* can oxidize glucose, gluconic, and 2-ketogluconic acids. The results of representative experiments on the oxidation of the acidic substrates are presented in Fig. 2. It will be seen that the oxidation of gluconic acid proceeds with the rapid consumption of 1 mole of O_2 per mole of substrate, while that of 2-ketogluconic acid involves only 0.5 mole of O_2. Following this initial phase, during which CO_2 is not evolved, a second, very slow reaction can be observed in which CO_2 is produced. The O_2 uptake and CO_2 production during this second stage represent an approximately 1:2 relationship. It must, however, be emphasized that the rate of O_2 consumption is not significantly different from that of the endogenous control solution, so that it is doubtful whether the process of CO_2 liberation is connected with substrate oxidation. It might easily represent a spontaneous decomposition of the primary oxidation product. In the course of time the solutions take on a brownish tinge.

Numerous experiments with such cell-free extracts have shown that the results are in agreement with those obtained with suspensions of "old" cells or of "young" cells in the presence of DNP. The chemical reactions of the oxidation products are also fully comparable. For the purpose of isolating or characterizing these products, the use of cell-free extracts is less advantageous than that of resting cell suspensions, largely because of the introduction with the former of a considerable amount of extraneous material. It should be emphasized, however, that the ease with which an active enzyme extract can be prepared should open the way to a characterization of the enzyme components.

Characterization of Main Oxidation Product—For the following studies the

oxidation product was prepared as a solution by permitting heavy suspensions of *A. melanogenum* in water to act on appropriate substrates, usually Ca gluconate (concentration 20 μM per ml. at pH 3.5 to 5.7), until the requisite amount of O_2 had been absorbed. The cells were then removed by centrifugation and the supernatant solution stored in a frozen state, or lyophilized and kept in a desiccator at low temperature.

The substance is very unstable; its solutions cannot be concentrated, even *in vacuo* at room temperature, without decomposition, which is always accompanied by the appearance of a brown color. It rapidly reduces the Luff-Schoorl alkaline copper reagent (14) and Benedict's solution in the

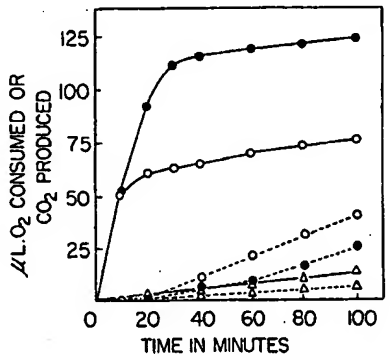


FIG. 2. Oxygen uptake and carbon dioxide evolution by cell-free preparations of *A. melanogenum*. The vessels contained 0.4 ml. of cell-free extract in 0.05 M phosphate at pH 5.9; 1.6 ml. of 0.05 M phosphate; 5 μM of substrate; total volume, 2.1 ml. ± 0.2 ml. 10 per cent KOH in center well; endogenous values not subtracted. Solid line, oxygen; dash line, carbon dioxide; ●, Ca gluconate; ○, Ca 2-ketogluconate; △, endogenous.

cold. It fails to give a positive orcinol test (15), nor does it react with ferric chloride even upon the addition of alkali. Ascorbic acid treated similarly produced its characteristic color with the latter reagent. The unknown oxidation product immediately reduces 0.1 N silver nitrate solution, a reaction typical of ascorbic and hydroxypyruvic (16) acids. Calcium 2-ketogluconate does not give this reaction.

From solutions obtained through the oxidation of Ca gluconate or Ca 2-ketogluconate with aged cells, followed by centrifugation and immediate large scale lyophilization of the centrifugate, there is obtained a pale yellow calcium salt. After several reprecipitations from a small volume of water by the addition of absolute alcohol, followed by drying *in vacuo*, a pale yellow friable powder reminiscent of Ca glutamate is obtained. Every effort to isolate this material as the free acid has been fruitless.

At neutral pH the calcium salt shows an absorption maximum at 305

mμ (Fig. 3). Raising the pH to 9 does not displace this maximum, although the optical density is somewhat diminished. Lowering the pH to 1 shifts the maximum to 280 mμ. Raising the pH of an acidified solution to 8 does not displace the peak back to 305 mμ; instead, the solution turns brown. It was observed that at the end of several hours the absorption of the solutions falls off, regardless of the pH of the medium, although there is no change in the wave-length of maximal absorption. Diminution of optical density with time due to oxidation occurs with ascorbic acid (17), while the shift of the maximum towards the lower wave-lengths upon acidification has been associated with enediol compounds such as ascorbic and dihydroxymaleic acids (18).

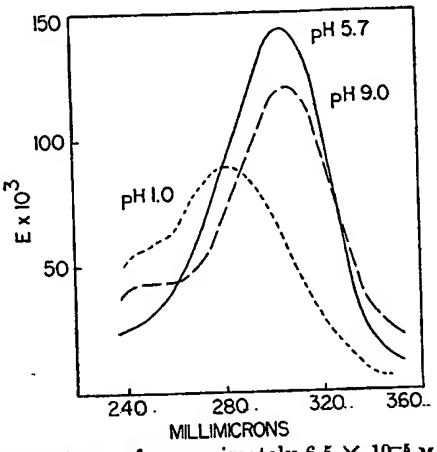


FIG. 3. Absorption spectrum of approximately 6.5 × 10^{-5} M Ca 2,5-diketogluconate in aqueous solution.

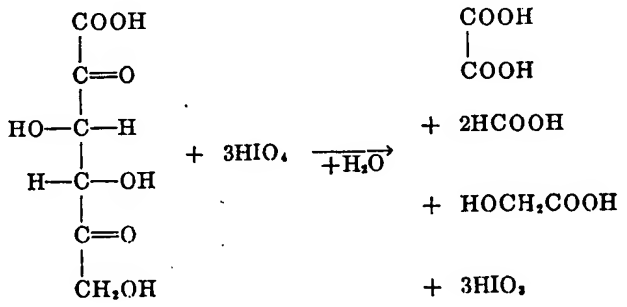
All of the reducing power of a solution of diketogluconic acid is absorbed by Duolite A-2 anion exchange resin, indicating that such neutral active reducing compounds as methylglyoxal or dihydroxyacetone are not produced in the oxidation of the substrates. Reducing power of the best preparations of the Ca diketogluconate (chromatographically pure) by the method of Stiles *et al.* (19) gave a value (with respect to glucose) of 58 per cent. 2-Ketogluconate had a reducing value of 86 per cent under identical conditions. The reported values for 2-ketogluconate and 5-ketogluconate are respectively 87 and 80 per cent (10). However, it might be expected that the competing action of warm alkali in the reducing mixture would rapidly destroy much of the diketogluconate molecule, thus lowering the amount available for copper reduction.

The presence of reactive carbonyl groups is substantiated by the rapid formation at room temperature of ill defined reaction products (osazones)

with phenylhydrazine and 2,4-dinitrophenylhydrazine. Although these presumptive osazones are soluble in acetone, ethyl acetate, dioxane, and to a lesser extent in benzene and toluene, and may even be extracted from these organic solvents by alkaline carbonate solutions, it has not been possible to obtain clean compounds by these procedures. With increased attempts at manipulation and purification, these derivatives become more amorphous and turn darker. Attempts to prepare a good semicarbazone, oxime, or hydrazone were also unsuccessful. Treatment with *o*-phenylenediamine failed to give insoluble condensation products; there was in addition no observable reaction with aniline citrate (20). These last observations suggest the absence of a carbonyl group at the 3 position.

Periodic Acid Oxidation—Treatment of the unknown presumptive diketogluconic acid with periodic acid resulted in the formation of oxalic and glycolic acids. No formaldehyde was detected among the products of this reaction. These moieties were identified by ascending partition chromatography by employing a modification of the methyl benzoate-*n*-propyl alcohol-formic acid-water (21) solvent with brom phenol blue as the developing agent, and by direct isolation from the reaction mixture. It is assumed that formic acid is the third product of this oxidation, although it was not isolated, nor could it be detected by the above solvent system. On the basis of an empirical formula $(C_6H_7O_7)_2Ca \cdot 2H_2O$ indicated by proximate analysis of the calcium salt, in 10 minutes 2.5 moles, and in 30 minutes 2.75 moles, of periodate were consumed per mole of compound.

The exact position of the two carbonyl groups can now be assigned to the molecule. The absence of formaldehyde after oxidation shows that the 5 position is a carbonyl group rather than a secondary hydroxyl; this eliminates the 3 and 4 positions as sites for the former functional group, and the conclusion is confirmed by the isolation of glycolic acid. The other carbonyl group in the molecule is assigned to the 2-carbon by virtue of its derivation from 2-ketogluconic acid and by the isolation of oxalic acid. The only diketogluconic acid which fits these experimental data is 2,5-diketogluconate.



Reduction of Diketogluconate to Gluconic Acid—In order to demonstrate unequivocally that the diketogluconate was indeed a 6-carbon entity, and to eliminate the outside possibility that the appearance of the products of the periodate reaction came from a fortuitous mixture of smaller fragments, attempts were made to reduce the compound. An aqueous solution of Ca diketogluconate was smoothly reduced to "gluconic acid" by the action of sodium borohydride. The products of the reaction were identified by paper partition chromatography (Solvent C). Starting with chromatographically pure Ca diketogluconate, the reduced reaction medium examined in this fashion exhibited (*a*) loss of the single typical acidic, aniline hydrogen oxalate-reactive spot and (*b*) the appearance of a non-reducing acidic spot with a totally different R_F from that of the starting material. The compound so produced was identical with authentic gluconic acid.[2]

Conversion of Glucose-1-C^{14} to 2,5-Diketogluconic Acid—Although all the evidence points to a direct oxidation of glucose, gluconate, and 2-ketogluconate to 2,5-diketogluconate, the possibility cannot be precluded that some rearrangement of the molecule occurred in this series of transformations. Accordingly, glucose-1-C^{14} was added to a suspension of *A. melanogenum* cells, aged 24 hours, in a Warburg vessel and oxidation was allowed to proceed. At the end of 70 minutes the rapid oxygen uptake ceased, and the slow evolution of gas began (presumably CO_2, since no KOH was present). At this point 48 per cent of the theoretical O_2 required for the total conversion of glucose to 2,5-diketogluconate was consumed. The preparation was immediately cooled to 5°, the cells were centrifuged, and the centrifugate analyzed by direct plating of 0.1 ml. aliquots of this reaction mixture and of the stock glucose solution on Alconox-rubbed aluminum planchets. The total activity of original glucose was 64,830 c.p.m. per ml., the total activity of the reaction mixture was 31,810 c.p.m. per ml. Conversion of glucose to soluble products (presumably to diketogluconate) was therefore 49 per cent.

Further examination of the distribution of this radioactivity among the reaction products was carried out chromatographically. Accurately measured aliquots, both of this reaction mixture and of the stock glucose-1-C^{14} which had been added as substrate, were spotted on Whatman No. 541 paper and irrigated with Solvent C. After careful removal of all the residual isobutyric acid, the sheets were sprayed with brom phenol blue to mark the acid spots, and test strips were sprayed with aniline hydrogen oxalate for reducing sugars (Fig. 4). The activity was determined by examining the

[2] If, as seems likely, the method of reduction produced four isomers of gluconic acid due to the non-stereochemical introduction of hydrogen into the 2 and 5 positions, these were not resolvable in this chromatographic system. The reduced product is therefore referred to as "gluconic acid."

paper with an end window monitor, the active areas being marked with pencil. The paper was then cut into 1 inch squares and the radioactivity of each square was determined by placing the paper on an aluminum planchet and assaying with a gas flow windowless counter. The dried cells were also spread into an area 1 inch square and counted as above. From the results (Table II), the following conclusions can be drawn: (*a*) All of the activity in the oxidation mixture was present in an acidic, reducing spot,

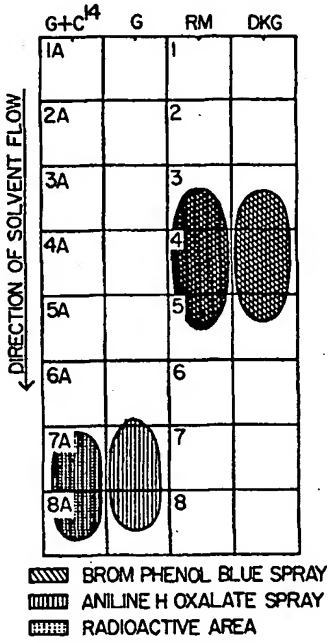


FIG. 4. Schematic appearance of chromatogram after enzymatic conversion of glucose-1-C^{14} to 2,5-diketogluconate-1-C^{14}. G-1-C^{14}, stock radioactive glucose; G, glucose control; RM, reaction mixture; DKG, diketogluconate control.

with an R_F value comparable to known diketogluconate on a test strip; (*b*) no glucose was left in the reaction mixture (apparently it had been assimilated into the cells). (*c*) The total radioactivity in the diketogluconate area (Spots 3, 4, and 5) corresponded to 49 per cent of the total activity of the original glucose (Spots 7A, 8A). This agrees well with the manometric data for the oxidation of glucose to diketogluconate. (*d*) 109 per cent of the total activity of the original glucose was recovered in the cells and the assayed paper squares of the oxidation mixture.

Degradation of 2,5-Diketogluconate-C^{14}—Attempts to remove the carboxyl carbon selectively presented several difficulties. Ceric sulfate oxidation in 10 N H_2SO_4 (22) and even in 1 N acid gave 3 moles of CO_2 per mole of Ca

diketogluconate. Although all the total activity of the starting glucose was recovered as CO_2, its specific activity was one-third of that calculated, so that no conclusions as to distribution of activity could be drawn from this experiment. Hydrolysis with 8 per cent H_2SO_4, used successfully for the degradation of ascorbic acid-1-C^{14} (23), failed to produce any CO_2.

TABLE II

Conversion of Glucose-1-C^{14} to 2,5-Diketogluconate-C^{14}

The Warburg vessel contained 2.7 ml. of stock glucose-1-C^{14}, 0.3 ml. of cell suspension in the side arm, and no KOH in the center well. Gas phase, air; shaken at 29°. 0.05 ml. aliquots of stock radioactive glucose and of the reaction mixture were pipetted onto the origins of the paper.

1 inch squares cut from glucose-1-C^{14} origin		1 inch squares cut from reaction mixture origin	
Square No.*	Activity†	Square	Activity†
7A	3268	2	32
8A	1188	3	247
Total.....	4456	4	1552
		5	167
		Total, Squares 3–5.....	1966
		6	153
		7	88
		8	42
		Total, Squares 2–8.....	2281
		Total activity of dried cells on planchet.....	2121
		Total activity recovered‡.....	4402

* Refer to Fig. 4 for the location of the squares.

† Expressed as total counts per minute per 1 inch square of chromatogram.

‡ Per cent radioactivity recovered $= \frac{4402 \times 3}{4456 \times 2.7} \times 100 = 109$ per cent.

Treatment with NaN_3 and H_2SO_4 (Schmidt reaction) at room temperature produced a black pigmentation without any CO_2 evolution. The problem was resolved by the use of an organic model decarboxylase, 3-aminooxindole, which smoothly decarboxylated the Ca salt in a phenol melt (24). The CO_2 produced in this manner had a specific activity of 320 c.p.m. per mg.; calculated, 370 c.p.m. per mg. This demonstrates that the oxidation of glucose through gluconate and 2-ketogluconate to 2,5-diketogluconate by *A. melanogenum* is a direct process.

EXPERIMENTAL

Preparation and Isolation of Ca 2,5-Diketogluconate—A. melanogenum was grown in five 3 liter Fernbach flasks, each containing 500 ml. of the medium described under "Methods." The cells were centrifuged, washed with tap water, and aged at 5° for 24 hours. 500 mg. of Ca gluconate·H_2O in 350 ml. of distilled water were added to a suspension of the aged cells contained in a modified atmospheric pressure-catalytic hydrogenation apparatus. The gas phase in the eudiometer was air. The mixture was stirred magnetically. 38 ml. of oxygen (76 per cent of theory) were taken up in 175 minutes, at which point gas uptake stopped and a slow gas evolution began. The mixture was centrifuged and the centrifugate was lyophilized to dryness. Paper chromatographic examination of this product showed it to be relatively pure diketogluconate. Only a single acidic and aniline hydrogen oxalate-reactive spot was apparent. Samples of Ca 2,5-diketogluconate prepared in this manner were always extremely electrostatic and difficult to handle. Accordingly, the lyophilized preparations were taken up in a small amount of water and precipitated by the addition of alcohol. After repeating this treatment several times, the precipitate was centrifuged and dried *in vacuo* over P_2O_5 at room temperature. It was not possible to obtain a good analysis on these samples. The best proximal analyses suggest an empirical formula Ca $(C_6H_7O_7)_2 \cdot 2H_2O$. This assumed formula was used in obtaining the approximate reducing values and periodate consumption data.

Periodic Acid Oxidation—100 mg. of Ca diketogluconate in 20 ml. of water were treated with 450 mg. of $HIO_4 \cdot H_2O$. Samples withdrawn from the reaction mixture and analyzed by ascending chromatography indicated the presence of oxalic and glycolic acids, besides iodic acids (ascending methyl benzoate-*n*-propyl alcohol-formic acid-water solvent, brom phenol blue spray). Addition of $CaCO_3$ to the solution, followed by concentration to small volume, precipitated the calcium salts of oxalic, iodic, and periodic acids. The filtrate was concentrated further, cooled, and alcohol was added to 70 per cent concentration by volume. Seeding with Ca glycolate produced a white gelatinous, semicrystalline precipitate, which was filtered off and dried. To 30 mg. of this Ca glycolate in 6 ml. of 50 per cent alcohol were added 50 mg. of *p*-brom phenacyl bromide, and the homogeneous mixture was refluxed 6 hours. Upon cooling, a first precipitate of excess reagent was removed; then concentration afforded plates of the *p*-brom phenacyl ester of glycolic acid. After recrystallization from water-alcohol, the product melted at 142–143°; mixed melting point with an authentic sample prepared as above, 143–144°. Both isolated and authentic esters showed the tendency of partially subliming at 125–126°.

To 1.1 gm. of the dried mixture of Ca oxalate, Ca carbonate, and inor-

ganic Ca salts were added 40 ml. of 1.5 N H_2SO_4. The $CaSO_4$ was removed and the filtrate was extracted with ether continuously for 24 hours. Upon evaporation of the ethereal extract to dryness, crystals of oxalic acid·$2H_2O$ remained. These were purified by sublimation; m.p. 185°; melting point of sublimed authentic oxalate·$2H_2O$, 186–187°; mixed melting point, 185–186°. The isolated material gave the specific aniline blue test (25) for oxalate.

Reduction of Diketogluconate to "Gluconate"—100 mg. of sodium borohydride in 2.5 ml. of distilled water were mixed with 25 mg. of chromatographically pure Ca diketogluconate in 0.5 ml. of water. An immediate vigorous bubbling of H_2 gas ensued, indicating reduction of the diketone. The reaction mixture was analyzed by chromatography (Solvent C).

3-Aminooxindole—Isatin oxime was prepared by the method of Schachat *et al.* (26) with some modifications. 37 gm. (0.25 mole) of isatin were slurried in 150 ml. of water and poured into a round bottom flask fitted with a stirrer. Upon the addition of 28 gm. of $(H_2NOH)_2SO_4$ (0.17 mole) in 150 ml. of water and 23 gm. of Na acetate·$3H_2O$ (0.17 mole), stirring was started and the mxture was heated to 60°. Within 2 hours the color had changed from deep red to light yellow. The product was collected by filtration and dried at 70° for 18 hours; melting point, sinters at 225°, decomposing sharply at 230–231°.

Catalytic hydrogenation at atmospheric pressure (27) reduced the isonitrosooxindole to the amine. A supercooled solution was prepared by dissolving 3.24 gm. (20 μM) of dried isatin oxime in 200 ml. of boiling glacial acetic acid and carefully cooling to room temperature. A suspension of freshly prepared palladium black in water was added and reduction was allowed to proceed by stirring magnetically. After 2 hours, 40 mM of hydrogen (100 per cent theory) were taken up and the reaction ceased. The clear yellow solution obtained after filtration of the catalyst began to turn cherry-red upon exposure to air. Addition of 25 ml. of 5 N HCl caused stabilization and reversion to the yellow color. Evaporation of this solution at reduced pressure gave white crystals of 3-aminooxindole hydrochloride. The compound was dried *in vacuo* over P_2O_5 and NaOH and was recrystallized from 80 per cent ethanol with the aid of Norit. A melting point determination on the micro stage showed that the amine·HCl turns pink at 125°, purple at 150°, and has an indeterminate decomposition point at about 195°.

Decarboxylation of Ca 2,5-Diketogluconic Acid-1-C^{14}—To 1.0 ml. of the radioactive diketogluconate solution obtained by the action of *A. melanogenum* upon glucose-1-C^{14} were added 100 mg. of carrier Ca diketogluconate. After gentle warming to effect solution, the mixture was taken down to dryness under a stream of nitrogen. 8 gm. of phenol were added and the whole was heated to a melt on a water bath. 35 mg. of 3-aminooxindole

were added and a stream of nitrogen gas was blown over the surface of the melt and passed through a 0.1 N $Ba(OH)_2$ bubbler to entrain CO_2 evolved. 15 minutes of heating at 85° imparted a clear yellow color to the mixture. The water bath was removed and gentle heating with a small yellow flame turned the solution a deep purple; concurrently CO_2 evolution began. The reaction was then allowed to proceed for an hour on the water bath. 26 mg. of $BaCO_3$ (yield 30 per cent) were produced by this procedure. A blank determination with all reagents except diketogluconate produced no CO_2. The $BaCO_3$ was centrifuged, washed with water and alcohol, plated on aluminum planchets, and counted in an end window counter.

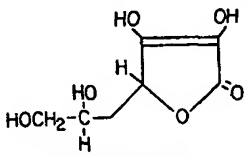
DISCUSSION

The establishment of the two carbonyl groups in the unknown gluconic acid derivative at the 2 and 5 positions by chemical means strengthened the prediction based on biochemical analogy that these would be the probable points of oxidative attack. Since 2-ketogluconate was oxidized to the same end-product as were glucose and gluconic acids, and was identified as an intermediate product in the oxidation of these compounds, it was logical to assume that one carbonyl occupied the 2 position. The configuration of the remaining 4-carbon entity is such that Bertrand's rule (28) requires the formation of a second carbonyl on carbon 5. It is true that this rule was derived from studies with the "sorbose bacterium" *A. xylinum*, but later studies, especially with *A. suboxydans*, have provided evidence in favor of the view that it applies to the activities of other "incomplete oxidizers" of the *Acetobacter* group. *A. melanogenum* may now be included among the organisms conforming to this principle, in so far as the final oxidation step is concerned.

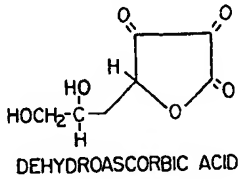
That 2,5-diketogluconic acid is unstable need not be surprising. It can be expected to undergo condensation reactions as well as decarboxylations readily; in this manner it is easy to regard it as the agent responsible for the gradual development of the brown color in cultures of the organism. While this has long been known to occur in glucose media, it has not heretofore been reported for cultures containing oxidation substrates other than glucose. Special experiments have established that cultures in gluconate and 2-ketogluconate media turn brown in time, the sooner and the more intense, the higher the concentration of the substrate.

At present little can be said about the possible metabolic significance of this curious oxidation product. Bernhauer and Riedl-Tumova (29) have reported the production of a "ketogluconic acid," not identical with either 2- or 5-ketogluconate, by the action of *A. melanogenum* on glucose. Their product may well be identical with the compound described here. A glance at the accompanying formula of 2,5-diketogluconic acid written in the lactone form shows its relationship to vitamin C; in fact, it can be regarded

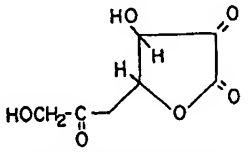
as a positional isomer of dehydroascorbic acid. It is possible that the formation of 2,5-diketogluconic acid is not restricted solely to *A. melanogenum*, but rather that this compound may play an intermediary rôle in



ASCORBIC ACID



DEHYDROASCORBIC ACID



2,5-DIKETOGLUCONIC ACID

those organisms which oxidize sugar directly rather than by the Embden-Meyerhof pathway.

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SUMMARY

1. In studies with "old" intact cells and cell-free extracts of *Acetobacter melanogenum*, it was found that glucose, gluconate, and 2-ketogluconate were oxidized to a common end-product. Its formation required the utilization of 1.5, 1.0, and 0.5 μM of O_2, respectively, per micromole of the substrates, and involved no CO_2 liberation. "Young" intact cells carried out a more complete oxidation with CO_2 evolution; in the presence of DNP their metabolism resembled that of old cells.

2. This end-product is unstable, especially at pH above 4.5, and easily gives rise to brown-colored decomposition or polymerization products. These are probably responsible for the characteristic appearance of cultures of *A. melanogenum* in glucose and gluconate media.

3. From the nature of its oxidation products on treatment with periodate, and from the fact that it yields "gluconate" on reduction, it has been inferred that this product is 2,5-diketogluconic acid.

4. With glucose-1-C^{14} as substrate, aged cells formed 2,5-diketogluconate as the sole radioactive product in the medium; one-half of the labeled carbon was found in the cells. Degradation of the radioactive diketogluconate recovered from this experiment showed conclusively that only the carboxyl group of this product contains the carbon isotope.

5. The experimental findings support the conclusion that *A. melanogenum* oxidizes glucose without preliminary splitting of the molecule as follows: glucose → gluconic acid → 2-ketogluconic acid → 2,5-diketogluconic acid.

Addendum—After completion of this investigation, Foda and Vaughn (30) reported 5-ketogluconic acid to be the end-product of maltose oxidation by *A. melanogenum*. In additional experiments our cultures of *A. melanogenum* oxidized maltose to 2,5-diketogluconic acid via 2-ketogluconic acid. No 5-ketogluconic acid was detected.

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